

CD49d and CD26 are Independent Prognostic Markers for Disease Progression in Patients with Chronic Lymphocytic Leukemia

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Introduction

Chronic lymphocytic leukemia (CLL) is a clonal expansion of small mature lymphocytes accumulating in blood, bone marrow, and lymphoid organs. CLL is characterized by extremely variable clinical courses with survivals ranging from one to more than 15 years [1]. Rai and Binet clinical staging systems were established for prognostic stratification of these cases [2,3]. However, there is a great variation in disease progression and survival of patients belonging to the same stage [4]. Adding simple clinical and laboratory parameters, such as age, sex, absolute lymphocytic count, lymphocytic doubling time (LDT) and serum beta2 microglobulin (β 2-MG) to clinical stage improved the prediction of overall survival (OS) and time to first treatment in early stage CLL [5]. Several serum markers such as CD23 and thymidine kinase were shown to have prognostic relevance in patients with CLL [6,7]. Also, several biological markers can predict disease progression and therapeutic outcomes in patients with early stage CLL including *IGHV* mutational status, ZAP-70, CD38 and cytogenetic abnormalities [8]. Recently an impressive array of novel effective therapies has been developed that hold the potential of increasing individualized treatments if patient risk could be accurately characterized. Unfortunately, the large number of novel prognostic markers in CLL, limited information on their independent prognostic value, and a lack of understanding of how to interpret discordant markers are still major barriers to integrate these in routine clinical CLL practice [9].

CD49d, an adhesion molecule belonging to integrin family, plays a critical role in leukocyte trafficking, activation, and survival. It mediates cell-to-extracellular matrix and cell-to-cell interactions, through binding with fibronectin or vascular cell adhesion molecule-1 (VCAM-1), respectively [10]. In addition to its adhesion functions, CD49d can affect B-cell survival via upregulation of Bcl-2 family members [11]. Some investigators reported that CD49d expression on B-CLL cells is lower than normal B cells, and is related to the presence of lymphadenopathy and disease stage [12]. Early studies suggested that CD49d expression may affect overall survival (OS) in CLL patients [13] as well as prognostic stratification of CLL [14,15]. However, little is published on the value of CD49d as an independent prognostic factor for OS and disease progression by multivariate analysis.

CD26 is a multifunctional type II cell surface glycoprotein that is widely expressed by T lymphocytes, natural killer (NK), epithelial, endothelial and acinar cells of many tissues [16]. CD26 expression is very low in B-cells; however, it is upregulated following *in vitro* activation [17]. CD26 has been suggested to play a role in the pathogenesis and progression of many types of tumors. Also, it may play a role in preventing apoptosis and metastasis as a result of its ability to bind extracellular matrix proteins [18]. It is highly expressed in aggressive subtypes of T-cell lymphomas [19]. However, fewer studies reported its constitutive expression in patients with B-CLL [20] and little had been published about its prognostic relevance in such patients. In this study, we will focus on the independent prognostic relevance of CD49d and CD26 expression in patients with chronic lymphocytic leukemia.

Patients and Methods

From April 2009 to April 2013, a total of 103 patients with newly diagnosed CLL were recruited from Mansoura oncology center. Informed consent was obtained from every patient for laboratory studies according to the guidelines of Committee of Medical Ethics of Mansoura University Hospitals. This study was approved by the Committee on Human Research at Mansoura University, Egypt. CLL diagnosis was based on NCI Working Group criteria and confirmed by a flow cytometry score >3 . Patients were selected to be newly diagnosed cases before receiving any treatment. The studied CLL patients composed of 70 males and 33 females with mean age \pm SD (59.51 ± 8.279) years. Lymphadenopathy was present in 99 (96.1%) cases, splenomegaly in 71 (68%), and hepatomegaly in 59 (57.3%) of patients. Cases were classified at diagnosis according to Binet and Rai staging systems. According to Binet classification, 36 (35.0%) were in Binet's stage A, 23 (22.3%) in stage B and 44 (42.7%) in stage C. While Rai classification comprised of 5 stages; 0 stage in 2 cases (1.9%), I in 28 (27.2%), II in 26 (25.2%), III in 43 (41.7%) and IV in 4 (3.9%) of cases. Median follow-up was 42 months with 31 patients progressed to a more advanced disease, 15 died and 88 living patients. The patients were investigated for expression of CD26 and CD49d on CLL cells in peripheral blood samples.

Sampling: specimen collection

- Three ml EDTA: one ml blood was collected for complete blood count by automated cell counter (Sysmex SE 9000). Two ml EDTA blood for immunophenotyping using a FACS calibur flow cytometer (Becton Dickinson, San Jose, CA).
- One ml serum for β 2-microglobulin (β 2-m) and LDH.

Analysis of CD26 and CD49d expression by flow cytometry

Expression of CD26 and CD49d were analyzed at time of diagnosis using a FACS calibur flow cytometer (Becton Dickinson, San Jose, CA). Three-color immunofluorescence by combining phycoerythrin (PE)-conjugated anti-CD26 or anti-CD49d monoclonal antibodies (mAbs) (BD Pharmingen, San Diego, CA, USA) with Peridinin-Chlorophyll-Protein-Cyanine-5.5 (PerCP-Cy5.5)-conjugated anti-CD19 mAbs (BD

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Received November 06, 2014; Accepted December 24, 2014; Published December 30, 2014

Citation: Ibrahim L, Elderiny WE, Elhelw L, Ismail M (2014) CD49d and CD26 are Independent Prognostic Markers for Disease Progression in Patients with Chronic Lymphocytic Leukemia. J Leuk 3: 173. doi:10.4172/2329-6917.1000173

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Biosciences, Franklin Lakes, NJ, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-CD5 mAbs (Becton Dickinson, Oxford, UK). The expression of each marker was reported as percentage of CD 5+ CD 19+ CLL cells displaying specific fluorescence intensity greater than 98-99% of the same cell population stained with control immunoglobulin. The best cutoff points for CD49d and CD26 expression that discriminated CLL patients from controls was sought by constructing receiver operating characteristic (ROC) curves. Regarding CD49d, the area under the ROC curve (AUC) was = 0.724, 95% CI= 0.626-0.822, P=0.005, Cutoff=30.4, sensitivity 55.3% and specificity 100%. While CD26, the area under the ROC curve (AUC) was = 0.827, 95% CI = 0.744-0.909, P<0.001, Cutoff=10.2, sensitivity 57.3% and specificity 100%).

The control of instrument calibration and the stability of instrument setup were made on a daily basis by immunophenotyping leukocyte subpopulations from fresh normal PB; further compensation controls were run on a weekly basis using reference fluorospheres (Calibrite beads; Becton Dickinson) [21,22].

Analysis of CD38 and ZAP 70 expression by flow cytometry

Samples were prepared using a three-color staining method by combining CD5-FITC, CD19-PerCP, and CD38- PE. B-CLL cells (CD5+/CD19+) were gated. The degree of CD38 expression in this gated population was expressed as percentage positivity. Percentages of ZAP-70-positive CLL cells are determined with negative threshold cutoff values set using ZAP-70-stained normal B-cells, as well as isotype control-stained B-CLL cells. The cutoff point for ZAP-70-positive in B-CLL cells was $\geq 20\%$ and for CD38-positive was $\geq 30\%$, respectively.

Definition of end points

Lymphocyte doubling is defined as progressive lymphocytosis with an increase of more than 50% over a 2-month period or lymphocyte doubling time (LDT) of less than 6 months. Factors contributing to lymphocytosis or lymphadenopathy other than CLL (e.g. infection) were excluded. Lymphocyte doubling time was measured from diagnosis to lymphocyte doubling, death or last follow up. Progression free survival is defined as the time from study entry until objective disease progression, first line treatment according to NCI Working Group guidelines, death or last follow-up. Overall survival (OS) was measured from diagnosis to last follow-up or death. All patients underwent first treatment at the time of documentation of progressive and symptomatic disease according to NCI Working Group guidelines.

Statistical methods

All of the statistical calculations were made using excel program and SPSS (statistical package for social science) program (SPSS, Inc, Chicago, IL) version 16. Qualitative data were presented as frequency and percentage. Chi square test was used to compare groups. Quantitative data were presented as mean and standard deviation. For comparison between two groups; student t-test, and Mann-whitney test (for non parametric data) were used. The associations between variables were tested by Spearman's correlation test. The best cut-off point for CD49d and CD26 expression was sought by constructing receiver operating characteristic (ROC) curves. Kaplan-Meier test was used for survival analysis and the statistical significance of differences among curves was determined by Log-Rank test. The Cox proportional hazards regression model was chosen to assess the independent effect of covariables on lymphocytic doubling time and progression free survival. N.B: P is significant if ≤ 0.05 at confidence interval 95.

Results

Expression of CD 49d and CD 26

Surface expression of CD49d and CD26 was investigated by three-color flow cytometry, and were reported as percent of CD 5 + CD19+ CLL cells expressing the antigen.

Regarding CD49d expression, a cutoff equal to 30% was used, so that values $\geq 30\%$ were considered high expression of CD49d, while those below this level were considered low. This is in same as training/validation strategy done by Bulian et al. [23,24] who found that 30% was the best cutoff to code CD 49d expression status. According to this cutoff value, 57 (55.3%) cases had high CD49d expression and 46 (44.7%) patients had low expression. For CD26 expression, a cutoff value of 10% was used. According to this cutoff value, 59 (57.3%) cases had high CD26 expression and 44 (42.7%) cases had low expression.

Figure 1 show the gating strategy used to quantify the expression of CD 26 by CLL cells. (a): forward scatter versus side scatter Lymphocytes were gated (R1). (b): CLL cells (CD19+ CD5 +, R2) were selected according to their phenotype. (c): Histogram shows CD 26 expression after CLL cells (R2) gating. CLL cells were plotted, and the cells in the upper left quadrant were quantified as CLL cells with a high level of CD 26.

The gating strategy for CD 26 positive and negative CLL cells. (a): Shows discrimination between CD26-positive (R2) and CD26-negative (R3) CLL cells. (b) Shows levels of these two subpopulations were compared using mean fluorescent intensity (MFI) values of cells located in gates R2 and R3 (Figure 2).

Relationship of CD49d and CD26 expression to clinical and laboratory characters of patients

High expression of CD49d and CD26 was significantly associated with splenomegaly (0.001, <0.001 respectively) and more advanced Binet and Rai stages of CLL (P=0.002, <0.001, 0.001, <0.001 respectively). CD 49d expression was significantly associated with hepatomegaly (P=0.001). CD49d^{high} and C26^{high} were associated with significant decrease in platelets count (p<0.001 for both), significant decrease in HB concentration (P=0.002, P<0.001 respectively), and significant increase in LDH versus low expression (P<0.001 for both). Surface expression of CD 49d and CD 26 was significantly correlated with CD 38 expression (r=0.651, P<0.001; r=0.374, P<0.001 respectively). Also, CD 49d and CD 26 was significantly correlated with ZAP 70 expression (r=0.339, P<0.001; r=0.253, P=0.010).

Prognostic relevance of CD49d and CD26 expression

As shown in Table 1, CD49d^{high} and CD26^{high} expression groups showed significantly higher number of cases with disease progression (P =0.001, 0.002 respectively). High CD26 expression group showed significantly higher number of cases who developed lymphocyte doubling (P=0.007).

Table 2 and Figure 3 show lymphocyte doubling time, progression-free survival and overall survival in patients according to CD49d positivity. Patients with high CD49d expression had significantly shorter time to develop lymphocyte doubling (15.8 versus 28.8 months) and significantly lower cumulative proportion at 24 months still without lymphocyte doubling (9.1 versus 66.7) (P<0.001). Patients with high CD49d expression had significantly shorter progression-free survival (16.4 versus 31.7 months) and significantly lower cumulative proportion at 24 months survived without progression (16.1 versus 87.8) versus those with low CD49d expression (P<0.001). Overall

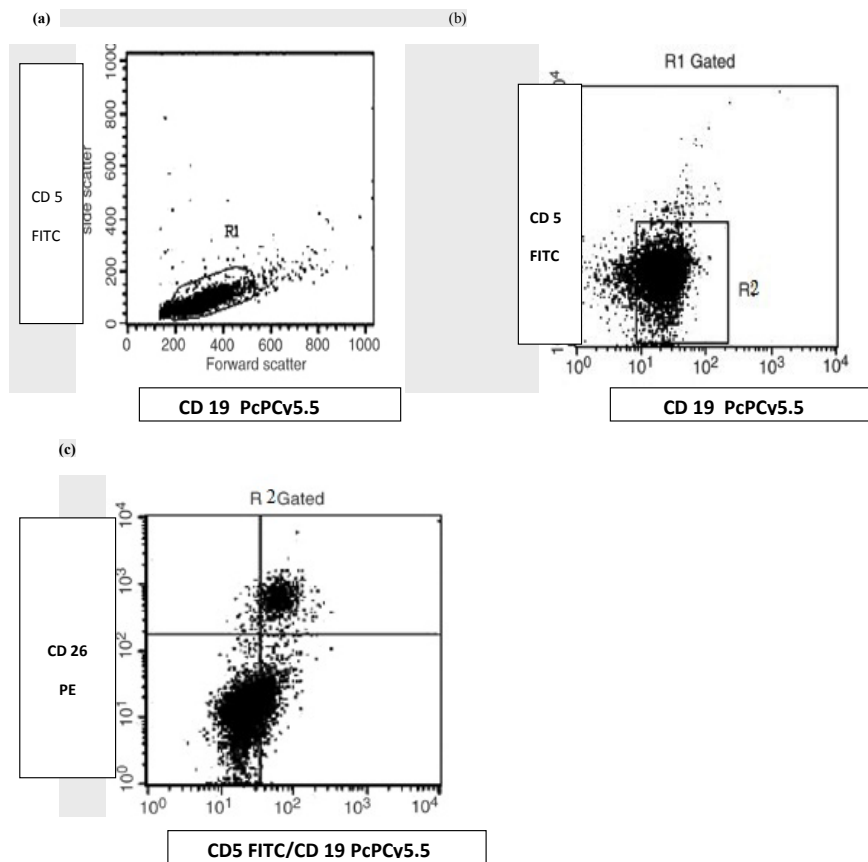


Figure 1: The gating strategy used to quantify the expression of CD 26 by CLL cells. (a): forward scatter versus side scatter Lymphocytes were gated (R1). (b): CLL cells (CD19+ CD5 +, R2) were selected according to their phenotype. (c): Histogram shows CD 26 expression after CLL cells (R2) gating. CLL cells were plotted, and the cells in the upper left quadrant were quantified as CLL cells with a high level of CD 26.

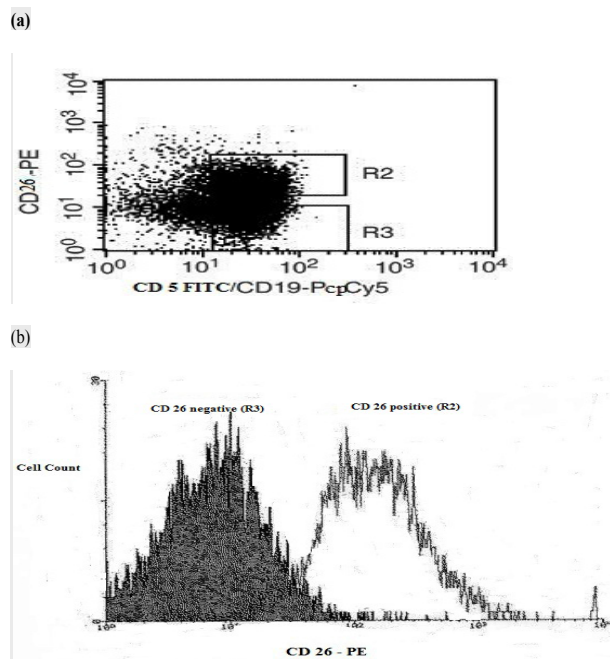


Figure 2: The gating strategy for CD 26 positive and negative CLL cells. (a): Shows discrimination between CD26-positive (R2) and CD26-negative (R3) CLL cells. (b): Shows levels of these two subpopulations were compared using mean fluorescent intensity (MFI) values of cells located in gates R2 and R3.

	CD49d ^{low} (n=46)		CD49d ^{high} (n=57)		P	CD26 ^{low} (n=44)		CD26 ^{high} (n=59)		P
	No	%	No	%		No	%	No	%	
Lymphocyte doubling	11	23.9	20	35.1	0.219	7	15.9	24	40.7	0.007
Progression to a more advanced stage	5	10.9	23	40.4	0.001	5	11.4	23	39	0.002
Lethal outcome	6	13	9	15.8	0.694	6	13.6	9	15.3	0.818

Table 1: Clinical outcome of patients according to CD49d and CD26 expression.

Survival	CD49d ^{low} (n=46)			CD49d ^{high} (n=57)				P Log Rank (Mantel-Cox)	CD26 ^{low} (n=22)			CD26 ^{high} (n=28)				p Log Rank (Mantel-Cox)
	Cumulative proportion (%)	Mean (months)	95% CI	Cumulative proportion (%)	Mean (months)	95% CI	Cumulative proportion (%)		Mean (months)	95% CI	Cumulative proportion (%)	Mean (months)	95% CI			
Lymphocyte doubling time	66.7	28.83	26.02 31.64	9.1	15.81	13.62 18.01	<0.001	75	30.5	27.21 33.78	15.4	17.69	15.11 20.27	<0.001		
Progression-Free Survival	87.8	31.7	30.06 33.35	16.1	16.45	14.62 18.28	<0.001	79.4	30.38	28.16 32.61	41.6	19.64	16.99 22.29	<0.001		
OS	81.5	30.57	28.39 32.74	83.5	28.64	26.33 30.95	0.303	77.6	30.09	27.63 32.54	83.5	28.64	26.33 30.95	0.519		

Cumulative proportion: at 24 months. 95%CI: Confidence interval at 95, OS: Overall survival.

Table 2: Clinical outcome and survival in patients according to CD49d and CD26 expression.

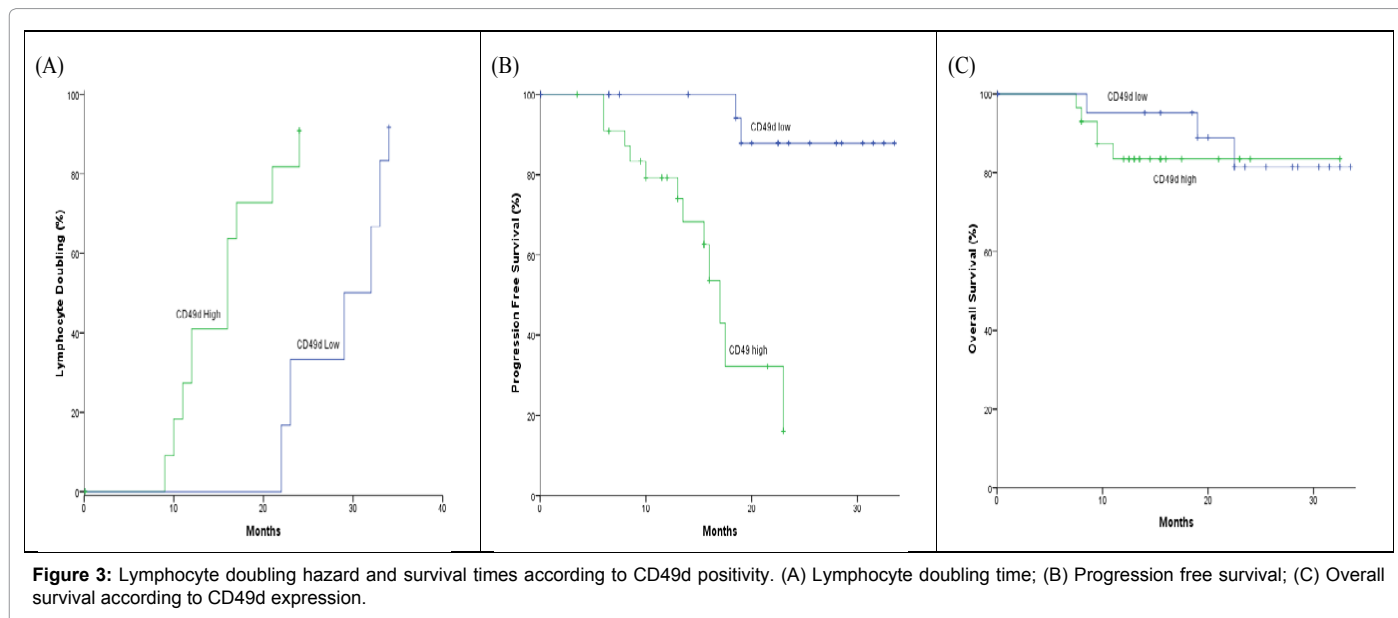


Figure 3: Lymphocyte doubling hazard and survival times according to CD49d positivity. (A) Lymphocyte doubling time; (B) Progression free survival; (C) Overall survival according to CD49d expression.

survival showed no significant differences between high and low expression of CD49d (P=0.538).

Table 2 and Figure 4 show lymphocyte doubling time, progression-free survival and overall survival in patients according to CD26 expression. Patients with high CD26 expression had significantly shorter time to develop lymphocyte doubling (17.7 versus 30.5 months) and significantly lower cumulative proportion at 24 months still without lymphocyte doubling (15.4 versus 75.0) versus those with low CD26 expression (p<0.001). Patients with high CD26 expression had significantly shorter progression-free survival (19.6 versus 30.4 months) and significantly lower cumulative proportion at 24 months survived without progression or treatment (41.6 versus 79.4) versus those with low CD26 expression (p<0.001). Overall survival showed no significant differences between high and low CD26 groups (P=0.519).

Prognostic relevance of combined expression of CD49d and CD26:

The combined expression of CD49d and CD26 was analyzed in

our CLL patients: 38 cases were CD49d^{low}/ CD26^{low} and 51 cases were CD49d^{high}/CD26^{high}, while the remaining 14 cases were discordant for the two markers. The concordant expression of both markers was statistically significant (P < 0.001). There was no significant difference between the three groups regarding lymphocyte doubling, progression free survival or Overall survival. However, on comparing CD49d^{high}/CD26^{high} versus CD49d^{low}/ CD26^{low}, there was significant difference between both groups regarding lymphocyte doubling and progression free survival but not Overall survival as shown in Figure 5.

Multivariate analysis for Lymphocyte doubling time

The value of CD49d and CD 26 as independent prognostic factors for lymphocyte doubling was assessed by multivariate analysis applying age, absolute lymphocyte count, Rai staging, LDH, expression of CD49d, CD26, CD 38 & ZAP 70 as covariates. Multivariate analysis revealed that high absolute lymphocyte count, high expression of CD49d, CD26, CD 38 and ZAP 70 are independent prognostic factors for developing lymphocyte doubling (P = 0.008, 0.049, 0.008, 0.030 and 0.043 respectively) (Table 3).

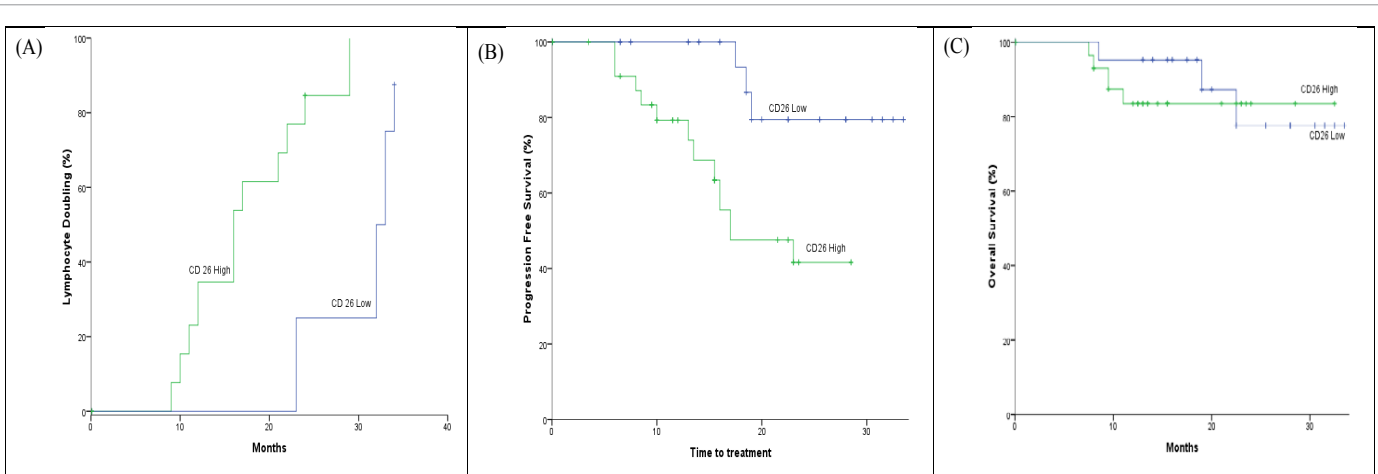


Figure 4: Lymphocyte doubling hazard and survival times according to CD26 positivity. (A) Lymphocyte doubling time; (B) Progression free survival; (C) Overall survival according to CD26 expression.

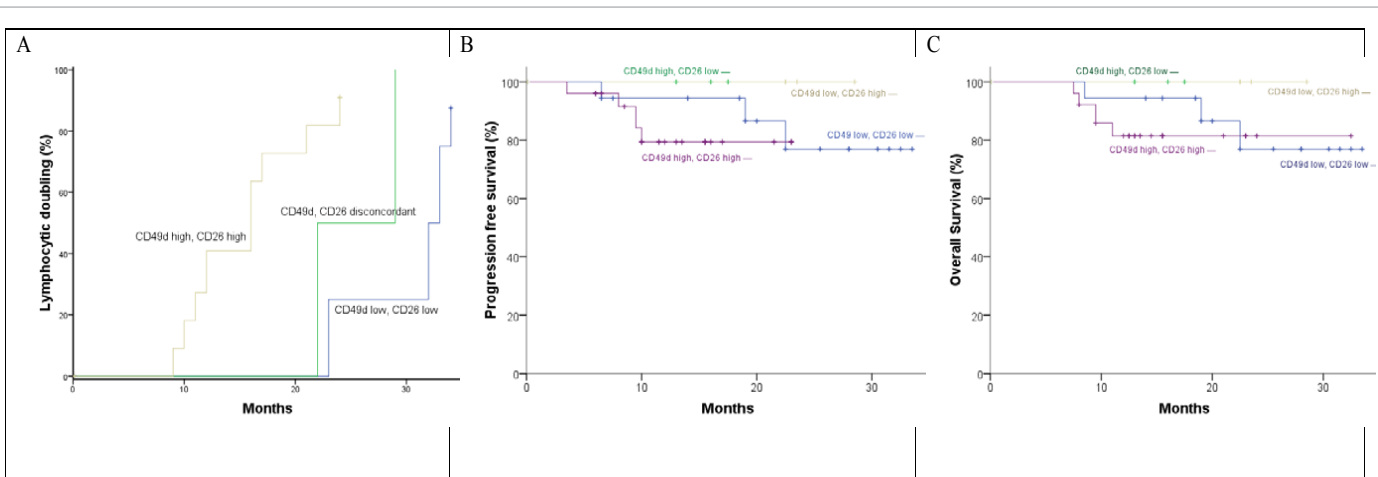


Figure 5: Prognostic value of combined expression of CD49d and CD26 in: (A) Lymphocyte doubling; (B) Progression free survival and (C) Overall survival.

Multivariate analysis for progression-free survival

The value of CD49d and CD 26 as independent prognostic factors for progression free survival was assessed by multivariate analysis applying age, absolute lymphocyte count, Rai staging, LDH, expression of CD49d, CD26, CD 38 and ZAP 70 as covariates. Multivariate analysis showed that high expression of CD49d, CD26, CD 38 and ZAP 70 are independent prognostic factors for progression-free survival ($P = 0.032, 0.048, 0.043$ and 0.029 respectively) (Table 3).

Discussion

Extreme clinical heterogeneity is one of the hallmark features of CLL. Despite the identification of genetic and phenotypic markers that correlate with prognosis, the biological basis of this clinical variability remains unclear. Progressive CLL is defined by expansion of the neoplastic clone and extravascular accumulation in lymphoid tissues, the bone marrow and other organs. Infiltration at these sites gives rise to the characteristic clinical picture of immune dysfunction, lymphadenopathy, splenomegaly and haematopoietic failure; features that correlate with survival [2,3].

The aim of the present study was to evaluate the role of CD49d and

CD26 as independent prognostic markers in CLL patients. In addition to overall survival, the present study used time to lymphocyte doubling and time to progression to a more advanced stage as indicators of disease kinetics [23]. By using univariate log-rank analysis, high expression of CD49d and CD26 were identified as adverse prognostic factors for lymphocyte doubling and disease progression. However, both markers were not significant for predicting overall survival in contrast with others [25-29]. The association between CD49d expression and time to progression and/or overall survival has been described by a number of research groups including, Gattei et al. [23], Shanafelt et al. [26], Rossi et al. [30], Bulian et al. [24]. The results of this study are consistent with previously published studies. The lack of association between CD49d and survival in this study could be related to the relatively small sample size and short follow-up of early stage patients. Early studies found higher levels of CD49d in advanced Rai stages than early stages [29] and others considered it an adverse prognosticator for CLL in univariate analysis [14,29,30].

Furthermore, multivariate analysis between clinical parameters and flowcytometry markers revealed that high expression of CD49d and CD26 are independent prognostic markers for lymphocyte doubling ($P=0.049, 0.008$ respectively) and for progression to a more advanced

Covariates	Lymphocyte doubling time				Progression-Free Survival			
	P	HR	95% CI		P	HR	95% CI	
Age	0.472	0.971	0.897	1.052	0.396	0.979	0.933	1.028
Rai	0.747	0.852	0.323	2.248	0.235	0.762	0.487	1.193
Absolute lymphocyte count	0.026	1.011	1.001	1.021	0.985	0.906	0.85	1.006
LDH	0.165	0.997	0.994	1.001	0.165	1.002	1	1.004
CD49d (high versus low)	0.049	3.602	0.628	4.644	0.032	3.982	1.123	6.118
CD26 (high versus low)	0.008	2.01	1.323	3.104	0.048	2.22	0.695	7.095
CD38	0.03	1.735	0.245	6.134	0.043	1.41	0.145	1.561
ZAP70	0.043	2.901	0.797	9.095	0.029	1.069	0.346	1.71

Table 3: Lymphocyte doubling time and Progression-Free Survival as dependent parameter studied with other covariates (multivariate analysis).

stage (P=0.032, 0.048 respectively). This is concordant with previous studies [23,28,30].

Previous studies described concordance among different prognostic markers such as between CD38 and both CD49d and ZAP-70 [31-33]. Additionally, many studies suggested that the use of an additional prognostic marker may be more useful than their individual use, such as combined analysis of CD38 and ZAP-70 or CD38 and CD49d [27]. The present study found a significant association between CD49d and CD26 in our CLL series, the finding which is similar to others [25,34]. Additionally, combined analysis of both markers was found to improve their power to predict the risk of lymphocyte doubling and disease progression but not overall survival. Bauvois et al. [20] have reported CD 26 constitutive expression in peripheral B-cells of patients with B-CLL. Cro et al. [25] demonstrated that CD26-expression significantly correlated with CD49d and CD38 in B-CLL cells, and there was also an association between CD26 and ZAP-70 or IgVH mutational status. These observations provide evidence for the expression of CD26 in B-CLL which, therefore, may be considered as a novel marker for B-CLL. Our result also in accordance with Bulian et al. [24] who turned out CD 49d at the top of other flowcytometric prognostic markers (Zap 70, CD 38) to stratify prognosis of CLL patients.

Many studies reported that CD49d and CD26 are surface markers whose expression can be easily investigated, highly reproducible, stable over time and stable in frozen samples [23,25]. Additionally, these markers can be easily evaluated by flow cytometry, which is a less expensive and widely used technique, and their combined use may be beneficial in borderline result of one marker. The determination of these markers should be recommended in all patients with B-CLL at diagnosis, and their reactivity could become a future routine panel for prognostic stratification.

Measurement of CD49d expression in CLL patients may have therapeutic implications. Anti-CD49d antibodies could impair migration and adhesion of leukaemic cells and overcome apoptotic resistance. Natalizumab, commercially available anti-CD49d antibodies, are approved by the Food and Drug Administration (FDA) for treatment of multiple sclerosis [33]. Therefore, use of anti-CD49d antibodies in combination with established chemotherapy or immunotherapy protocols may give a promise in CLL therapy.

In conclusion, surface expression of CD49d and CD26 are independent adverse prognostic markers in patients with CLL at different stages. Therefore, evaluation of CD26 and CD49d expression is suggested as a part of routine future panel for prognostic stratification of CLL patients at diagnosis.

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